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# The Genes for Three Xylan-Degrading Activities from *Bacteroides ovatus* Are Clustered in a 3.8-Kilobase Region

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**Genes coding for three xylan-degrading activities, xylanase, xylosidase, and arabinosidase, were simultaneously cloned from the colonic anaerobic organism *Bacteroides ovatus*. The genes for the three enzymes were located on a 3.8-kilobase *Eco*RI genomic insert and were cloned by using plasmid pUC18. All three activities were expressed in *Escherichia coli* JM83, and all were cell associated. Expression of the xylanase gene was independent from expression of the xylosidase and arabinosidase genes, whereas expression of the latter two genes appeared to be coordinated. Restriction endonuclease analysis of the arabinosidase and xylosidase genes and partial purification of these enzyme activities from *E. coli* suggested that these activities were catalyzed by a bifunctional protein or two proteins of very similar molecular weight. All three enzyme activities were regulated in *B. ovatus* in response to the carbon source used for growth. This is the first report of the cloning and expression of *B. ovatus* genes.**

The fiber consumed in the human diet is primarily composed of plant cell wall polysaccharides, including cellulose, hemicelluloses, and pectins. These polysaccharides cannot be degraded by digestive enzymes produced by humans and other animals. However, bacteria inhabiting the colon produce enzymes capable of degrading these compounds. Diets high in fiber are increasingly being recommended for a variety of health reasons, and the products of degradation or fermentation of these fiber polysaccharides by the colonic microflora may be utilized by humans. Some of the microorganisms that may be involved in degradation of these polysaccharides have been identified; however, little is known about the biochemistry of these degradation processes by colonic bacteria.

Salyers et al. (21) previously demonstrated that *Bacteroides* species, which constitute about 20% of the colonic bacterial population, can ferment a variety of plant polysaccharides, including amylose, xylan, and pectin. Subsequently, Salyers et al. (20) reported on the degradation of xylan, the major component of hemicelluloses, by two *Bacteroides* species, including *B. ovatus*. Since then, there have been few reports on the degradation of xylan by human colonic bacteria. The objectives of the current study were to determine the levels of xylanolytic enzymes produced by *B. ovatus* and isolate the gene(s) coding for xylanase activity. Fortuitously, we were also able to clone the genes coding for arabinosidase and xylosidase activities on the same DNA fragment that encoded xylanase activity. These genes are the first to be cloned from *B. ovatus*.

## MATERIALS AND METHODS

**Bacterial strains and media.** *B. ovatus* V975, a spontaneously rifampin-resistant mutant of strain VPI 0038-1, was obtained from Francis Macrina, Virginia Commonwealth University, Richmond, Va. For isolation of DNA, *B. ovatus* was grown anaerobically on medium containing 37 g of brain-heart infusion (BHI; BBL Microbiology Systems, Cockeysville, Md.), 250 mg of hemin, 2 g of sodium carbonate, 0.5 g of cysteine, and 1 mg of resazurin per liter under an atmosphere of 20% $CO_2$–80% $N_2$. For growth on different carbon sources, *B. ovatus* was grown on RGM medium, a complex yeast extract-Trypticase-salts medium (9). *Escherichia coli* JM83 (24) was grown on LB medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl per liter) or LB supplemented with ampicillin (75 μg/ml) (LBA) when selecting for transformants. LBA agar plates containing 3.5 mg of 4-*O*-methyl-D-glucurono-D-xylan–Remazol brilliant blue R (RBB-xylan; Sigma Chemical Co., St. Louis, Mo.) per ml were used for screening transformants for the cloned xylanase gene.

**Cloning procedures.** Plasmid pUC18 (24) was used for cloning with *E. coli* JM83 as the cloning host. Genomic DNA from *B. ovatus* and *E. coli* was isolated as described by Saito and Miura (19). *B. ovatus* genomic DNA was partially digested with *Eco*RI, and DNA fragments of 3 to 10 kilobases (kb) were recovered from a low-melting-point agarose gel by using an Elutip-d column (Schleicher & Schuell, Keene, N.H.) according to the manufacturer's instructions. The digested DNA was ligated with T4 DNA ligase into *Eco*RI-digested, dephosphorylated pUC18, and the ligated plasmid DNA was transformed into competent *E. coli* JM83 (13). Transformed cells were screened on LBA-RBB-xylan plates, and xylanase-positive clones were identified by clear zones around the colonies. Positive clones were picked and restreaked to confirm clearing of the RBB-xylan. *E. coli* clones were also screened for xylosidase and arabinosidase activities on LBA agar plates containing 20 μg of 4-methylumbelliferyl-β-D-xyloside or 4-methylumbelliferyl-α-L-arabinofurnoside (Sigma Chemical Co., St. Louis, Mo.) per ml, respectively. The colonies were examined under UV light (365 nm) for fluorescence, indicating the presence of enzyme activity.

For recovery of plasmid DNA, cultures of *E. coli* were grown on LBA to the stationary phase, and plasmid DNA was isolated by the modified alkaline lysis procedure described by Morelle (14). Large-scale preparations were purified by CsCl gradient centrifugation.

**DNA analysis.** DNA was digested with restriction endonucleases according to the manufacturer's instructions, electrophoresed through agarose gels in 89 mM Tris–68 mM phosphoric acid–2 mM EDTA, and stained with ethidium bromide. For hybridization with biotinylated probes, the

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DNA was transferred to nitrocellulose by the method of Southern (22). Gel-purified DNA fragments were labeled with a random primed DNA-labeling kit (Boehringer Mannheim, Indianapolis, Ind.) and [11-biotin]dUTP according to the manufacturer's instructions. Hybridizations and staining with streptavidin-biotin-alkaline phosphatase were carried out with a DNA detection kit (Bethesda Research Laboratories, Gaithersburg, Md.) according to the manufacturer's instructions. Several subclones of the genomic insert were generated by using exonuclease III digestion as described by Henikoff (8).

**Protein and enzyme analyses.** *B. ovatus* was grown in RGM containing 0.2% carbon source to mid-log phase ($A_{660}$ of approximately 0.6, 1-cm light path), centrifuged at 10,000 × *g* at 4°C for 10 min, and washed twice with 50 mM potassium phosphate (pH 6.8)–10 mM dithiothreitol (DTT). Cells were suspended to 5% of the original culture volume in the same buffer and broken by a single passage through a French pressure cell at 12,000 lb/in$^2$. The broken-cell suspension was centrifuged at 15,000 × *g* for 20 min at 4°C. The supernatant fluid (crude extract) was recovered and used for enzyme assays and protein analyses. In addition, for some experiments the crude extract was subjected to ultracentrifugation at 150,000 × *g*, 4°C, for 1 h. The supernatant fluid was recovered and termed the cytoplasmic fraction. *E. coli* strains carrying the vector or hybrid plasmids were grown in LBA to an $A_{600}$ of about 0.8 and processed as described above.

Xylanase activity was determined by monitoring release of sugars from oatspelt xylan (2 mg/ml) with orcinol as described before (9) and xylose as the standard. One unit of enzyme activity was defined as the amount of enzyme that catalyzed formation of 1 μmol of xylose equivalents in 1 h at 37°C. Arabinosidase and xylosidase activities were measured by spectrophotometrically monitoring the release of ρ-nitrophenol at 405 nm from ρ-nitrophenyl-α-L-arabinofuranoside and ρ-nitrophenyl-β-D-xyloside (Sigma Chemical Co., St. Louis, Mo.), respectively (9). One unit of activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of ρ-nitrophenol in 1 h at 37°C. Protein concentrations were estimated by the dye-binding assay of Bradford (4) with the commercial Bio-Rad Laboratories (Richmond, Calif.) reagent with fraction V of bovine serum albumin used as the standard. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (10). The stacking gel was 4% and the resolving gel was 10% acrylamide. Proteins were stained with Coomassie brilliant blue R-250. SDS-PAGE molecular weight standards were purchased from Bio-Rad Laboratories (Richmond, Calif.).

**Partial purification of arabinosidase and xylosidase.** Arabinosidase and xylosidase activities from *E. coli* JM83(pOX1) were partially purified by phenyl-agarose chromatography. Briefly, the crude cell extract prepared from *E. coli* JM83(pOX1) was subjected to ultracentrifugation at 150,000 × *g* for 1 h at 4°C. The supernatant fluid was recovered and maintained on ice while being adjusted to 1.0 M ammonium sulfate (approximately 19% saturated). This preparation was then centrifuged at 10,000 × *g* for 20 min at 4°C, and the supernatant fluid containing the enzyme activities was recovered. The fluid was applied to a phenyl-agarose column (2.5 by 1.5 cm) equilibrated with buffer A (50 mM sodium phosphate, 0.1 mM DTT, 1.0 M ammonium sulfate [pH 6.8]) at room temperature. The column was washed for 30 min with buffer A at a flow rate of 1 ml/min, followed by a linear gradient from 1.0 to 0.0 M ammonium sulfate in buffer A over 120 min. Column fractions (2 ml) were collected and assayed for arabinosidase and xylosidase activities. Peak fractions were pooled for further enzyme assays and analysis by SDS-PAGE.

TABLE 1. Specific activities of xylanolytic enzymes from *B. ovatus* grown on various carbon sources

| Carbon source | Xylanase sp act (U/mg) | Fold increase[a] | Arabinosidase sp act (U/mg) | Fold increase | Xylosidase sp act (U/mg) | Fold increase |
|---|---|---|---|---|---|---|
| Glucose | 0.47 | | 0.45 | | 0.018 | |
| Fructose | 0.58 | 1.2 | 0.35 | 0.8 | 0.011 | 0.6 |
| Xylose | 0.80 | 1.7 | 0.67 | 1.5 | 0.009 | 0.5 |
| Arabinose | 2.19 | 6.2 | 8.28 | 8.3 | 0.015 | 0.8 |
| Oatspelt xylan | 23.5 | 50 | 2.64 | 5.9 | 1.80 | 100 |

[a] Increase (or decrease) over activity in glucose-grown cells.

**Degradation of xylooligosaccharides by cloned xylosidase.** Degradation of the natural substrates xylopentaose (X5) and xylobiose (X2) by the phenyl-agarose-purified xylosidase from *E. coli* JM83(pOX1) was examined. The enzyme was incubated with X2 or X5 at 37°C in 50 mM sodium phosphate–0.1 mM DTT, pH 7.0, for 5 h. The assay contents were then frozen and concentrated by lyophilization, and the residue was suspended in distilled water. The products were separated by thin-layer chromatography on Whatman silica gel plates (K-5; 250-μm particles) developed with nitroethane-ethanol-water (1:3:1, vol/vol/vol; S. L. Evans and R. J. Stack, manuscript in preparation). The products were visualized with *N*-(1-naphthyl)ethylenediamine spray (3).

**Materials.** Restriction enzymes, T4 DNA ligase, plasmid pUC18, 1-kb DNA ladder, [11-biotin]dUTP, and isopropylthio-β-galactoside were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). Oatspelt xylan was obtained from Sigma Chemical Co. (St. Louis, Mo.). Calf intestinal alkaline phosphatase was purchased from Boerhinger Mannheim Biochemicals (Indianapolis, Ind.). Nitrocellulose BA-85 (0.45-μm pore size) was obtained from Schleicher & Schuell (Keene, N.H.). All other chemicals were of reagent grade or better.

## RESULTS

**Induction of enzymes in *B. ovatus*.** The specific activities of xylanase, xylosidase, and arabinosidase in crude extracts of *B. ovatus* cells grown on RGM medium containing various carbon sources were determined (Table 1). All three activities increased significantly when cells were grown on oatspelt xylan compared with glucose. Xylanase, arabinosidase, and xylosidase activities increased 50-, 6-, and 100-fold, respectively, with growth on oatspelt xylan versus glucose. Little changes in these activities occurred following growth on fructose or, interestingly, on xylose. In contrast, growth on arabinose resulted in a sixfold and eightfold increase in xylanase and arabinosidase activities, respectively. However, xylosidase activity was not altered by growth on arabinose.

**Cloning of multiple *B. ovatus* genes.** A genomic library of *B. ovatus* DNA was prepared in *E. coli* JM83 carrying pUC18. The transformed bacteria were screened on LBA-RBB-xylan plates for xylanase activity. Initially, six clones of approximately 4,000 colonies screened were identified as being xylanase positive by clear halos around the colonies. Colonies of strain JM83 containing pUC18 did not clear the

TABLE 2. Specific activities of xylanolytic enzymes in various *E. coli* JM83 clones and *B. ovatus*

| Strain and plasmid (substrate) | Sp act (U/mg) | | |
|---|---|---|---|
| | Xylanase | Arabinosidase | Xylosidase |
| *B. ovatus* (glucose) | 0.47 | 0.45 | 0.018 |
| *E. coli* (LBA) | | | |
| pUC18 | 0.00 | 0.00 | 0.00 |
| pOX1 | 5.50 | 4.90 | 16.70 |
| pOX30 | 0.10 | 4.70 | 14.80 |
| pOX1:3G2 | 8.00 | 3.00 | 6.60 |
| pOX10 | 12.90 | 0.00 | 0.00 |

RBB-xylan plates. After secondary screening, one clone continued to be xylanase positive. This *E. coli* clone, containing a recombinant plasmid termed pOX1, was grown and assayed for various enzymatic activities (Table 2). The expressed xylanase activity was about 10-fold higher than that observed with glucose-grown *B. ovatus* cells. Surprisingly, high levels of both xylosidase and arabinosidase were also detected in the crude extract of the clone. None of these activities were detected in the culture fluid.

**DNA and enzyme analyses.** Plasmid DNA was isolated from the *E. coli* JM83(pOX1) clone and subjected to restriction endonuclease mapping. The plasmid contained a 3.9-kb *Eco*RI insert. The location of the genes coding for xylanase, xylosidase, and arabinosidase activities was determined by subcloning various fragments of the insert, or by exonuclease III deletion, and plating the transformed *E. coli* on methylumbelliferyl substrates or RBB-xylan (Fig. 1). By using exonuclease III digestion towards the *Hin*dIII site of the insert, one subclone was obtained that reduced the size by about 700 bp. While this subclone (pOX1:3G2) expressed all three activities (Fig. 1), the arabinosidase and xylosidase activities were reduced by at least 40% and the xylanase activity was increased by 50% (Table 2). Removal of another 200 bp (i.e., deletion of the *Eco*RI-*Hin*dIII fragment) yielded a subclone (pOX10) that lost both xylosidase and arabinosidase activities, but the xylanase activity was twofold greater than that expressed in the initial clone (pOX1).

Deletion of the 0.8-kb *Kpn*I-*Eco*RI fragment from the other end of the insert did not affect xylanase activity, but xylanase was lost when the larger 1.4-kb *Pvu*II-*Eco*RI fragment was deleted. Xylanase activity was also lost when about 700 bp were removed beyond the *Hin*dIII site (Fig. 1). These data indicate that the xylanase gene is located approximately between 1.5 and 3.5 kb on the map in Fig. 1, while the xylosidase and arabinosidase genes appear to be located around the 1.0-kb mark.

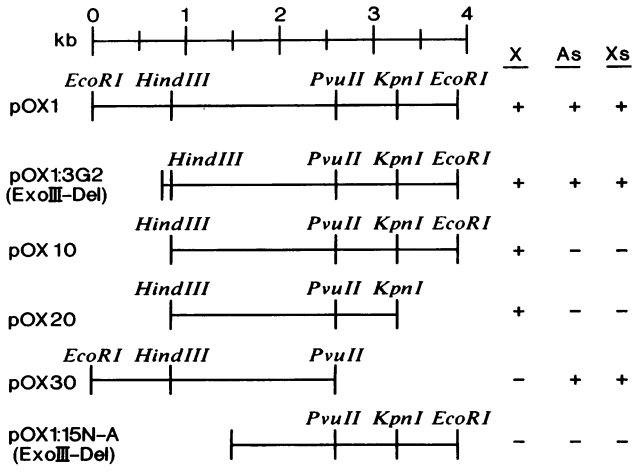


FIG. 1. Restriction endonuclease analysis of 3.9-kb genomic insert for locating sites of the xylanase (X), xylosidase (Xs), and arabinosidase (As) genes. Presence (+) or absence (−) of activity is indicated. ExoIII-Del, Exonuclease III-generated deletion.

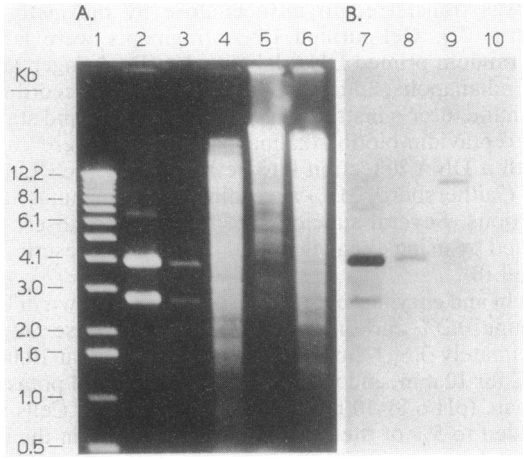


FIG. 2. Southern hybridization analysis of *B. ovatus* and *E. coli* genomic DNA digests with random-primed DNA synthesized from the 3.9-kb *Eco*RI insert from pOX1. (A) Photograph of 0.8% agarose gel stained with ethidium bromide. (B) Detection of hybridizing DNA with alkaline peroxidase-streptavidin conjugate following DNA transfer to nitrocellulose. Lane 1, Molecular size markers; lane 2, 0.4 μg of *Pst*I-digested pOX1; lanes 3 and 7, 0.010 μg of *Eco*RI-digested pOX1. Lanes 4 through 6 and 8 through 10 contain 5 μg of genomic DNA: lanes 4 and 8, *B. ovatus Eco*RI digest; lanes 5 and 9, *B. ovatus Bam*HI digest; lanes 6 and 10, *E. coli* JM83 *Eco*RI digest.

**Southern hybridization analysis.** In order to confirm that the cloned DNA was isolated from *B. ovatus* and no apparent rearrangement occurred during the cloning, the cloned 3.9-kb DNA fragment was labeled and hybridized with digested genomic DNA isolated from *B. ovatus* and *E. coli* JM83 (Fig. 2). The same 3.9-kb *Eco*RI fragment cloned into pUC18 was also observed in the *Eco*RI genomic digest from *B. ovatus* (lane 8), and a single *Bam*HI band was also noted (lane 9). No hybridization was observed with the digested *E. coli* DNA (lane 10). These results indicate that only one copy of these cloned genes is present in the chromosome. The second band hybridizing in lane 7 (2.7 kb) is probably due to contamination of the isolated 3.9-kb probe DNA with pUC18 DNA, which would also be labeled during random-primed DNA synthesis.

**Partial purification of arabinosidase and xylosidase.** The results of the restriction endonuclease mapping suggested that the arabinosidase and xylosidase activities were encoded by two genes that were very close together or that the two activities were associated with one protein. To clarify these possibilities, the arabinosidase and xylosidase activities were partially purified from JM83(pOX1) crude extracts. The purification involved sequential ultracentrifugation, recovery of supernatant fluid, and addition of ammonium sulfate to 1.0 M, followed by phenyl-agarose column chromatography. Both activities were found to copurify and elute from the phenyl-agarose column in the same fractions. The peak fractions were combined, and these proteins plus other

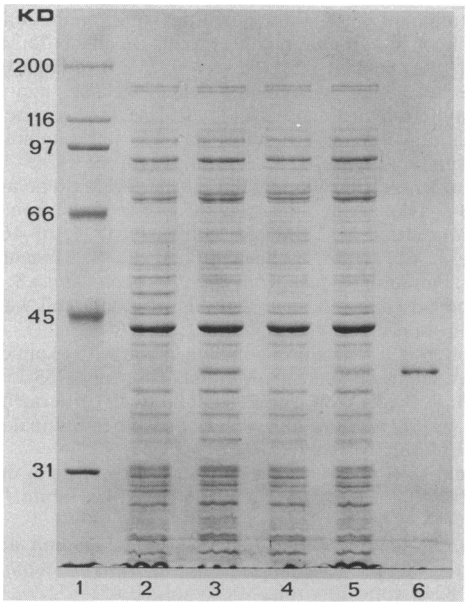


FIG. 3. SDS-PAGE analysis of cytoplasmic fractions from *E. coli* JM83 containing pOX1 or various subclones, and the purified arabinosidase-xylosidase from JM83(pOX1). Lanes 2 to 5 contain 20 μg of protein, and lane 6 contains 1 μg of purified enzyme. Lane 1, Molecular size markers (sizes shown in kilodaltons); lane 2, JM83(pUC18); lane 3, JM83(pOX1); lane 4, JM83(pOX10); lane 5, JM83(pOX30); lane 6, purified arabinosidase-xylosidase.

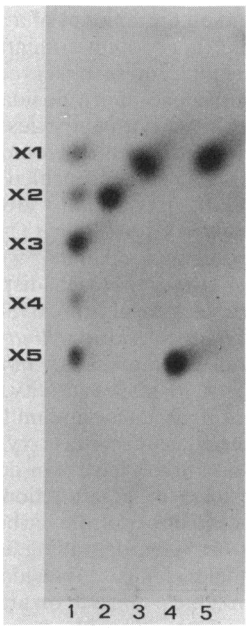


FIG. 4. Thin-layer chromatography analysis of degradation products from xylooligosaccharides by xylosidase purified from *E. coli* JM83(pOX1). Conditions are described in Materials and Methods. Lane 1, Xylooligosaccharide standards; lane 2, xylobiose (X2) without enzyme; lane 3, xylobiose with enzyme; lane 4, xylopentaose (X5) without enzyme; lane 6, xylopentaose with enzyme.

cytoplasmic fractions were analyzed by SDS-PAGE (Fig. 3). The cytoplasmic fraction from *E. coli* JM83(pOX1) (lane 3) contained a new protein band of about 38,000 molecular weight that was not present in JM83(pUC18) (lane 2). This protein was not present in JM83(pOX10) (lane 4), which had also lost both arabinosidase and xylosidase activities (Table 2, Fig. 1). However, this 38,000-$M_r$ protein was present in JM83(pOX30) (lane 5), which had lost only xylanase activity. SDS-PAGE analysis of the phenyl-agarose-purified enzymes showed a highly enriched protein that comigrated with the new 38,000-molecular-weight protein seen in the cytoplasmic fractions (lane 6). The enriched protein appeared to be at least 90% pure. It should be noted that no new additional protein bands were observed in extracts from clones expressing xylanase activity.

Since neither ρ-nitrophenyl nor methylumbelliferyl derivatives are natural substrates for the xylosidase, we wanted to confirm that the cloned xylosidase could degrade natural xylooligosaccharide substrates. Both xylobiose (X2) and xylopentaose (X5) were incubated with the phenyl-agarose-purified xylosidase, and the products were separated and identified by thin-layer chromatography (Fig. 4). Both X2 and X5 were degraded to xylose (X1), indicating that the enzyme is a true xylosidase. In addition, the purified enzyme was also tested with ρ-nitrophenyl-α-D-glucoside on the chance that the enzyme may be a nonspecific glycosidase. The enzyme had no activity on this substrate.

## DISCUSSION

This report describes the isolation and characterization of genes coding for xylanase, arabinosidase, and xylosidase activities from *B. ovatus*. These are the first genes to be cloned from *B. ovatus*, and all three are expressed in *E. coli*. The genes were cloned on one 3.9-kb genomic insert by using the plasmid pUC18.

The xylanase expressed in *E. coli* was capable of degrading both RBB-xylan and oatspelt xylan. The expression of this gene appears to be independent of the expression of the xylosidase and arabinosidase genes. Analysis of crude extracts from *E. coli* expressing only the xylanase gene revealed no new proteins (Fig. 3), despite a 10-fold increase in xylanase activity over that found from *B. ovatus* grown on glucose (Table 2). However, from restriction analysis we have determined that the gene resides in a region of about 2 kb (from about 1.5 to 3.5 kb on the map in Fig. 1). Therefore, the maximum subunit molecular weight of the encoded protein could be about 70,000. This would be reasonable in view of the reported sizes of xylanase from other bacteria (24,000 to 145,000 $M_r$ [15, 17]).

The simultaneous isolation of the xylosidase and arabinosidase genes was fortuitous, and their expression in *E. coli* has turned out to be quite revealing. As demonstrated by data from restriction endonuclease mapping (Fig. 1) and assay of activities in the resulting subclones (Table 2), the activities appear to be coordinately expressed and independent of the xylanase gene expression. In the clone pOX1:3G2, derived from exonuclease III digestion of the insert, both activities decreased at least 40%. The activities then disappeared altogether in the clone pOX10, despite a difference of only several hundred bases. Analysis of the cytoplasmic fraction from *E. coli* JM83(pOX1) expressing the activities demonstrated the appearance of a new protein at about 38,000 molecular weight (Fig. 3). The protein was missing from *E. coli* JM83 containing pOX10. In *E. coli* JM83 containing pOX1:3G2, the new protein appeared to be smaller, suggesting that the deletion extended into the coding portion of the gene (data not shown).

The two activities expressed in JM83(pOX1) were found to copurify through the ultracentrifugation, ammonium sulfate precipitation, and phenyl-agarose chromatography

steps. This resulted in an enrichment of a single protein to at least 90% purity, and this protein comigrated with the new 38,000-molecular-weight protein observed from clones expressing both arabinosidase and xylosidase activities (Fig. 3). These data suggest that both activities may reside within a single bifunctional protein, which would be a rather novel enzyme. Xylosidases have been purified from *Clostridium acetobutylicum* (11), *Bacillus pumilus* (16), *Bacillus coagulans* (6), and *Thermomonospora fusca* (1), and all are multisubunit enzymes ranging in molecular weight from 130,000 to 224,000. None of these enzymes display arabinosidase activity. Arabinosidases range from 65,000 to 310,000 $M_r$ and have been purified from *C. acetylbutylicum* (12), *Bacillus subtilis* (23), and *Ruminococcus albus* (7). Similarly, these enzymes lack xylosidase activity. While there are other examples of bifunctional (5) and multifunctional (2, 18) proteins, the *B. ovatus* arabinosidase-xylosidase would be the first example of one involving xylan-degrading enzymes.

The possible presence of a bifunctional protein is also interesting in light of the effect of the carbon source used for growth of *B. ovatus* on xylan-degrading activities (Table 1). The data indicates that xylanase, xylosidase, and arabinosidase are inducible enzymes. Growth on arabinose resulted in an eightfold increase in arabinosidase specific activity, but had no effect on xylosidase activity. Growth on oatspelt xylan, however, resulted in a 100-fold increase in xylosidase activity and a 6-fold increase in arabinosidase activity. When the cytoplasmic fractions from *B. ovatus* grown on arabinose or oatspelt xylan were subjected to phenyl-agarose chromatography, one peak of arabinosidase activity eluted in the same fractions from both samples. A second peak of arabinosidase activity eluted with the xylosidase activity from oatspelt xylan-grown cells, which was not present in the arabinose-grown cells (data not shown). Moreover, application of these activities to a fast protein liquid chromatography Mono-Q column separated the xylosidase-arabinosidase activities from the other arabinosidase activities (data not shown). These results suggest that there may be a second gene coding for an arabinosidase in addition to the arabinosidase-xylosidase enzyme. The presence of a second arabinosidase could explain why the arabinosidase activity in *B. ovatus* is consistently higher than the xylosidase activity (Table 1), whereas in the *E. coli* clones expressing both activities, the reverse was observed (Table 2). Further work will be needed to completely purify and characterize the activities in *B. ovatus*. We are beginning to investigate the structure and regulation of these genes by DNA sequencing and targeted chromosome inactivation of the genes in *B. ovatus*.

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